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Brain and Pituitary Immunocytochemistry of Carassin in the Goldfish, *Carassius auratus*: A New Neurohormone Peptide?

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ABSTRACT—Carassin is a 21-amino-acid tachykinin-related peptide originally isolated from the goldfish brain. Carassin-immunoreactive (ir) perikarya were restricted to the nucleus preopticus periventricularis (NPP); immunoreactive perikarya were distributed sparsely in the rostral and caudal NPP, and were comparatively more in the lateral areas of the mid-NPP. Most perikarya showed thick axonal processes that extended into the rostral area of the organum vasculosum lamina terminalis (OVLT) and terminated extensively in the vicinity of cells and blood capillaries in its ventral area below the preoptic recess; the OVLT may play a role in release of the carassin-ir material into the general circulation. The axonal terminals diminished caudally and were almost absent in the posterior region of the OVLT. Most epithelial cells of the olfactory organ were carassin-ir positive. Several fibers showing carassin-ir were also present in the olfactory bulb and are presumed to originate from the olfactory epithelium. Carassin-like ir granules were found in some cells of the proximal pars distalis (PPD) of the pituitary gland. Adjacent sections reacted with growth hormone (GH) and gonadotropin (GtH) antibodies, revealed that carassin coexists with GtH in a small percentage of cells. Further, there is sexual dimorphism in the nature and distribution pattern of carassin-ir granules in the PPD. In males, GtH cells contained a few carassin-ir granules; whereas, in females, GtH cells frequently had clusters of carassin-ir granules. These carassin-containing granules may participate in auto- and/or paracrine regulation of the pituitary. The ir-perikarya of the NPP may influence hypophysial function through a multisynaptic pathway. The functions of carassin in goldfish remain to be investigated.

INTRODUCTION

The mammalian nervous tissue contains at least five related tachykinins, substance P, neurokinin A, neurokinin K, and neuropeptide γ derived from the preprotachykinin A gene (Carter and Krause, 1990) and neurokinin B derived from the preprotachykinin B gene (Bonner *et al.*, 1987). Tachykinins play a role in an array of biological actions including pain transmission, vasodilation, smooth muscle contraction, secretion of saliva, bronchiole constriction, activation of the immune system, and participation in neurogenic inflammation and neurological diseases (Reviews: Maggio, 1988; Maggio and Mantyh, 1994). Tachykinins also function as neuromodulators and possess preferentially excitatory roles in the central nervous system (Review: Kow and Pfaff, 1988). Tachykinins have been isolated in pure form from the brain, gut and/or skin of chicken (Conlon *et al.*, 1988), amphibians (Kow and Pfaff, 1988; Waugh *et al.*, 1995), a reptile (Wang *et al.*, 1992), elasmobranchs (Conlon *et al.*, 1986; Conlon and Thim, 1988; Waugh *et al.*, 1994, 1995) and two species of lamprey (Waugh *et al.*, 1994). However, among the teleosts, isolation and purification of tachykinins was performed only in goldfish (Conlon *et al.*, 1991), rainbow trout, *Oncorhynchus mykiss* (Jensen and Conlon, 1992; Jensen *et al.*, 1993), and cod, *Gadus morhua* (Jensen and Conlon, 1992). Carassin is a 21-amino-acid tachykinin-related peptide isolated from the goldfish brain that belongs to the kassinin subfamily of tachykinins (Conlon *et al.*, 1991). Carassin has 57% structural homology to the mammalian tachykinin neuropeptide g (Conlon *et al.*, 1991) and is a homologue of peptides isolated from rainbow trout and hammerhead shark, *Sphyrna lewini* (Waugh *et al.*, 1995).

The brain distribution of the tachykinin substance P was studied in several mammalian (Kanazawa *et al.*, 1984) and teleostean species rainbow trout (Vecino *et al.*, 1989), goldfish (Sharma *et al.*, 1989), sea bass, *Dicentrarchus labrax* (Moons *et al.*, 1992), electric fish, *Apteronotus leptorhynchus* (Weld and Maler, 1992). With the exception of the study on electric fish (Weld and Maler, 1992), all the previous studies on fish have not dealt with the substance P-ir in the pituitary gland. Similarly there is no information on the brain distribution of other tachykinins in teleosts.

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The distribution of carassin immunoreactivity (ir) in the brain and pituitary of goldfish was investigated in the present study, using antisera raised against carassin.

MATERIALS AND METHODS

Goldfish of either sex (weight, 20-25 g) were purchased from local dealers in Nagpur, India, during the month of July when the gonads were in a regressed state. The fish were maintained in aquaria under normal photoperiod (13 hr light + 11 hr dark) and fed commercial fish food *ad libitum*. The fish were deeply anesthetized with 0.05% tricaine methanesulfonate before killing. The brain and pituitary gland of six males and six females were fixed for 24 hr in aqueous Bouin's fluid, transferred and subjected to several changes of 70% ethanol to wash out picric acid. After dehydration, the tissues were embedded in paraffin. Serial sections of the brains were prepared in transverse and sagittal planes (8-10 μm thickness), whereas the pituitaries were sectioned in sagittal plane(4-6 μm thickness).

Immunocytochemical study

Antiserum to synthetic carassin (Peninsula Laboratories, Inc., Belmont, CA, USA) was produced in rabbits. Carassin was conjugated to bovine thyroglobulin, by reacting with carbodiimide and hydroxylamine (Skowsky and Fisher, 1972). The conjugated peptide was initially injected with Freund's complete adjuvant at multiple subcutaneous and intramuscular sites in a domestic rabbit. Booster injections of conjugated peptide and Freund's incomplete adjuvant were given at four monthly intervals, and the rabbit bled and serum collected at the fifth month.

The sections were deparaffinized, hydrated and immunocytochemically reacted using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) based on the avidin-biotin-peroxidase complex (ABC) method with some modifications. The sections were treated with 0.3% H_2O_2 in methanol for 40 min to deactivate endogenous peroxidase activity and washed in 0.01M phosphate buffered saline I (PBS containing 0.9% NaCl) at pH 7.5 for 20 min. They were then incubated in PBS I containing BSA, gelatin and NaN_3 for 30 min. After a 10 min wash in PBS I, the sections were incubated in diluted (1:40) normal goat serum for 30 min. After blotting excess serum from the slides, sections were incubated for 20-24h at 4°C in a humidified chamber with antiserum raised against carassin at 1:180 dilution. The slides were washed in PBS I for 10 min and incubated in biotinylated goat anti-rabbit antibody for 30 min. After a 10 min wash in PBS I, the sections were incubated with ABC reagent diluted in PBS II (PBS containing 2.92% NaCl) for 40 min. The slides were washed three times in PBS I, each wash lasting 10 min. The sections were reacted in dark for 5-6 min in Tris buffer diluted with distilled water (1:1) containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide. The slides were rinsed in tap water for 5 min, counterstained with hematoxylin - eosin to identify brain areas, dehydrated in graded alcohol series, cleared in xylene and mounted in Permount.

To identify the carassin-ir cells in the proximal pars distalis, adjacent sections of the pituitary gland were reacted with growth hormone (GH) and gonadotropin (GtH) antisera at dilutions of 1:6000 and 1: 10,000 respectively. These antibodies were raised at the laboratory of RE Peter and the antisera have been extensively validated for specificity in radioimmunoassays for GH (Marchant *et al.*, 1989) and GtH-II (Van Der Kraak *et al.*, 1993), respectively. Subsequently, the serial sections of the pituitary gland were reacted for carassin, GH and GtH, counterstained with hematoxylin - eosin, photographed and traced on transparent sheets. These were matched in order to examine the occurrence of coexistence of carassin with GH or GtH. Nomenclature for brain nuclei was according to Peter and Gill (1975).

To verify the specificity of the carassin-ir staining, three control procedures were adopted: (i) incubation of sections with PBS instead of primary antibody, (ii) incubation with serially diluted primary antibody, and (iii) incubation of sections with antibody that was preabsorbed for 2 hr at room temperature with 20 μg/ ml of carassin. The neuronal diameters were measured using a calibrated eyepiece graticule.

RESULTS

Brain

Carassin immunoreactive somata and fibers were not found in brain or pituitary sections incubated with normal goat serum in place of primary antibody, or in sections reacted with antibody that was preabsorbed with carassin. The third control set in which sections were reacted with serially diluted primary antibody showed a gradual reduction and final abolition of the carassin-like ir.

The carassin-ir perikarya were limited to the nucleus preopticus periventricularis (NPP) of the rostral preoptic area. The anterior-most ir cell bodies were isolated small perikarya (diameter 8-10 μm) near the rostral border of the preoptic recess (Fig. 1A). These perikarya had thick, long axonal processes (Fig. 1B). There was an increase in number of ir somata at the midlevel of the NPP (Fig. 1C). Most carassin-ir somata at this level were located laterally, and were small (diameter, 8-12 μm), while a few were comparatively large (diameter, 18-20 μm) and showed prominent, thick axonal processes. In the posterior NPP, the carassin-ir perikarya were small in size (diameter, 7-10 μm) and sparse (Fig. 1D),

The rostral area of the organum vasculosum lamina terminalis (OVLT) had extensive carassin-ir fiber innervation (Fig. 1A) and the processes encircled the somata and blood capillaries of the OVLT (Fig. 1E). In the midarea of the OVLT, marked dorsally by the merger of the preoptic recess with the third ventricle, only a few carassin-ir fibers were found in the ventral zone (Fig. 1C). The caudal region of the OVLT, located anterior to the optic chiasma, was almost devoid of carassin-ir fibers (Fig. 1D). The carassin-ir fibers of the OVLT are presumed to arise from the carassin-ir perikarya in the NPP.

Olfactory organ

Most epithelial cells of the olfactory organ showed carassin-ir positive material (Fig. 2A). Several fibers showing carassin-ir were also present in the granule cell layer of the olfactory bulb (Fig. 2B) and are presumed to originate from the olfactory epithelium.

Pituitary gland

A few cells of the proximal pars distalis (PPD) showed carassin-ir granules in male and female goldfish (Fig. 3A-D). No carassin-ir was found in the rostral pars distalis, pars intermedia or neurohypophysial fibers. Adjacent sections reacted with GtH revealed that carassin-ir is colocalized in a small percentage of GtH cells of the proximal pars distalis (Fig. 3A,B). In males, one or two carassin-ir granules appeared in GtH cells, and as a result carassin-ir granules appear to be sparsely distributed in the PPD of males (Fig. 3C). On the other hand, in female fish most of the carassin-ir granules

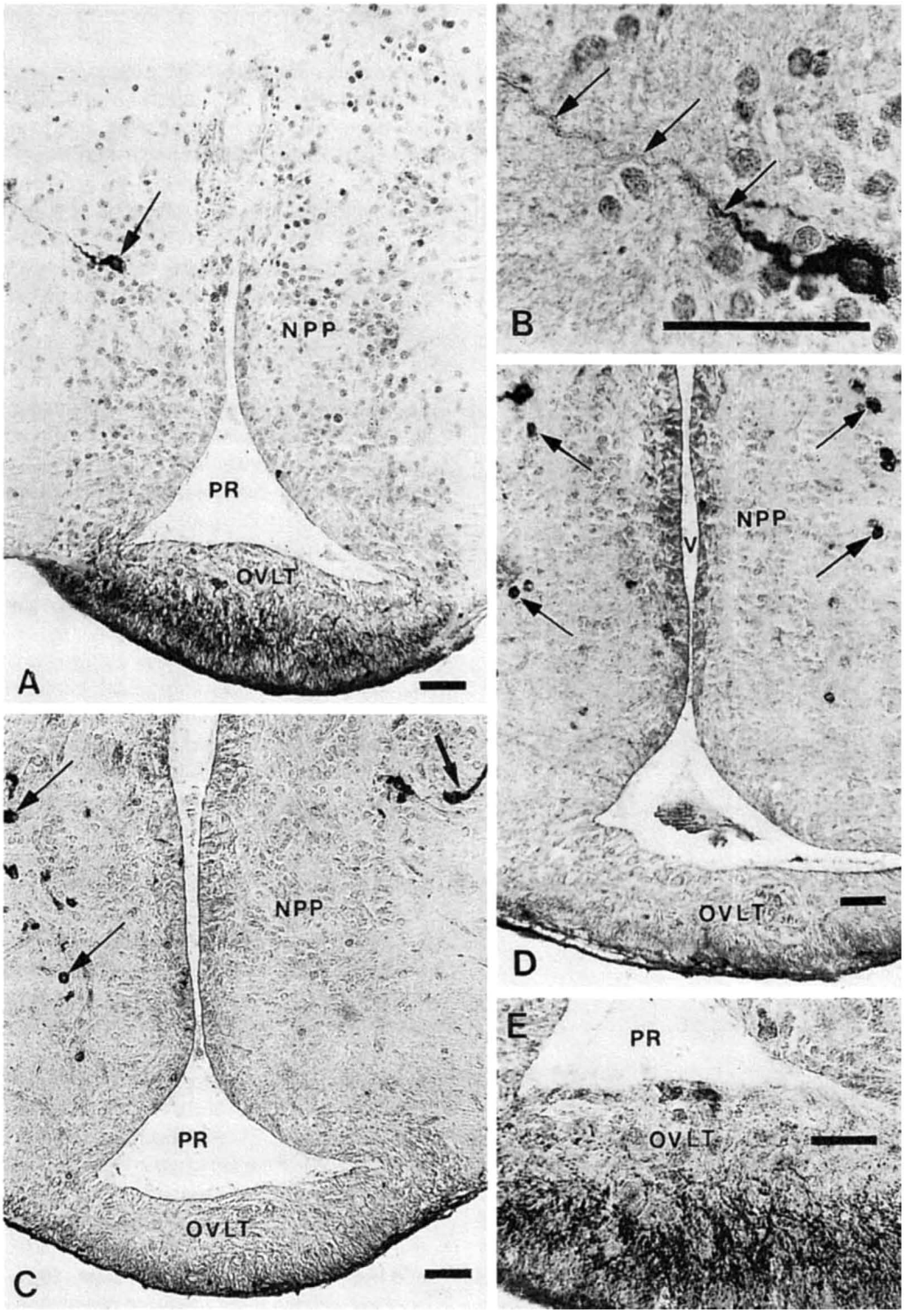

Fig. 1. Transverse section of goldfish brain passing through different levels of the preoptic area (counterstained with hematoxylin - eosin). A. An isolated carassin-ir neuron (arrow) in the nucleus preopticus periventricularis (NPP) near the rostral border of the preoptic recess (PR). Note the extensive fiber innervation of the rostral area of the organum vasculosum lamina terminalis (OVLT). B. A magnified view of the carassin-ir neuron shown in A. Note the thick and long axonal process (arrows). C. Section through the mid-level of the NPP showing several neurons (thin arrows) located laterally and only a few carassin-ir fibers in the ventral zone of the OVLT. Also note a cell body with thick axonal process (thick arrow). PR, preoptic recess. D. Section through the caudal region of the OVLT showing a few small carassin-ir cells in the NPP. Note that the OVLT at this level is devoid of carassin-ir fibers. V, third ventricle. E. A high power view of the OVLT located below the preoptic recess (PR) showing carassin-ir fibers around the somata and blood capillaries of the OVLT. Scale bars = 40 μm.

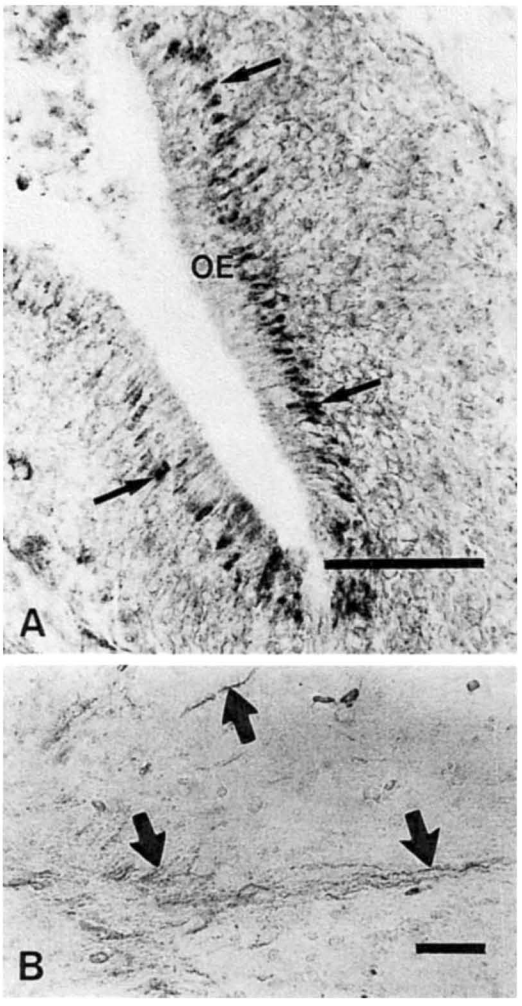

Fig. 2. Sagittal sections of the olfactory organ and bulb. A. Olfactory epithelium (OE) showing carassin-ir material (arrows) in the epithelial cells. B. Olfactory bulb showing carassin-ir fibers (arrows) in the granular cell layer. Scale bars = 40 μm.

appear as clusters in GtH cells (Fig. 3D).

DISCUSSION

This is the first study on the brain distribution of carassin-ir. Our results revealed carassin-ir perikarya in a single nucleus of the goldfish brain, namely the NPP. In addition, a dense network of carassin-ir fibers was found in the OVLT of the preoptic area. The carassin-ir fibers found in the OVLT are presumed to originate form the carassin-ir perikarya found in the NPP. As the OVLT is a circumventricular organ (McKinley *et al.*, 1990), our results suggest that carassin may be released into the general circulation. The neurohormonal functions that carassin might serve via the general circulation are not known. It should be noted that this is a homologous study, in that carassin was isolated from goldfish brain.

The NPP of goldfish and other teleosts has been shown to contain a number of neuropeptides and neurotransmitters, for example dopamine (Kah *et al.*, 1984), galanin (Cornbrooks and Parsons, 1991), somatostatin (Sas and Maler, 1991), cholecystokinin (Himick and Peter, 1994), bombesin/gastrin-releasing peptide (Himick and Peter, 1995), gamma amino-butyric acid (Martinoli *et al.*, 1990), gonadotropin-releasing hormone (Kah *et al.*, 1986; Kim *et al.*, 1995) and growth hormone-releasing hormone (Rao *et al.*, 1996). Retrograde transport studies have shown that the NPP cell bodies innervate the pituitary gland in the goldfish (Anglade *et al.*, 1993) and other teleosts (Johnston and Maler, 1992; Prasada Rao *et al.*, 1993). Although NPP is a large nucleus, carassin-containing perikarya were comparatively few and widely distributed, and represent only one small subpopulation of cells. Perhaps carassin-containing neurons have a neuromodulator function by innervating other NPP neurons. Notably, carassin-ir fibers were not observed in the pituitary, suggesting that there is no direct innervation of the pituitary by the NPP carassin-ir neurons. In the catfish (*Clarias batrachus*), the perikarya of the subventricular grey, that are comparable with the large OVLT cell bodies of goldfish, also innervate the adenohypophysis (Prasada Rao *et al.*, 1993). If a similar OVLT-hypophysial system exists in the goldfish, the extensive terminations of carassin-ir fibers around somata of the rostral OVLT provides an indirect means for carassin to influence pituitary activity.

Although a carassin-ir fiber pathway to the pituitary was not identified, carassin-ir granules in the PPD cells of the pituitary of males and females were observed. These carassin-ir granules were localized to GtH cells and are presumed to be produced by these cells. Carassin may therefore play a role in auto- and/or paracrine regulation of certain PPD hormones (see Schwartz and Cherny, 1992). Further studies are required to elucidate the functional significance of the sexually dimorphic distribution of carassin-ir in GtH cells, and their relationship with the hypothalamic perikarya.

The presence of carassin in epithelial cells of the olfactory organ is a novel finding. The presence of carassin-ir fibers in the olfactory bulb suggests some modulatory or transmitter-like role for this peptide in olfactory function.

The immunocytochemical distribution of the related tachykinin peptide substance P has been studied in goldfish (Sharma *et al.*, 1989) and other teleosts (Vecino *et al.*, 1989; Weld and Maler, 1992). Sharma *et al.* (1989) observed substance P-ir in the perikarya in the NPP, NPO, three different areas of the telencephalon and several mid- and hind-brain nuclei in goldfish. Similarly, a wide distribution of substance P-ir was demonstrated in rainbow trout (Vecino *et al.*, 1989) and the electric fish (Weld and Maler, 1992). These results contrast with the more restricted distribution of carassin-ir in the goldfish, and indicate a high level of specificity in the distribution of tachykinins in goldfish and other teleosts.

Our immunocytochemical findings indicate that carassin is produced by neurons in the NPP and produce an extensive carassin-ir fiber network in the OVLT. Carassin is presumably released to the general circulation at the OVLT, suggesting that it functions as a neurohormone. Carassin may also have neuromodulator actions on NPP and on OVLT neurons, which

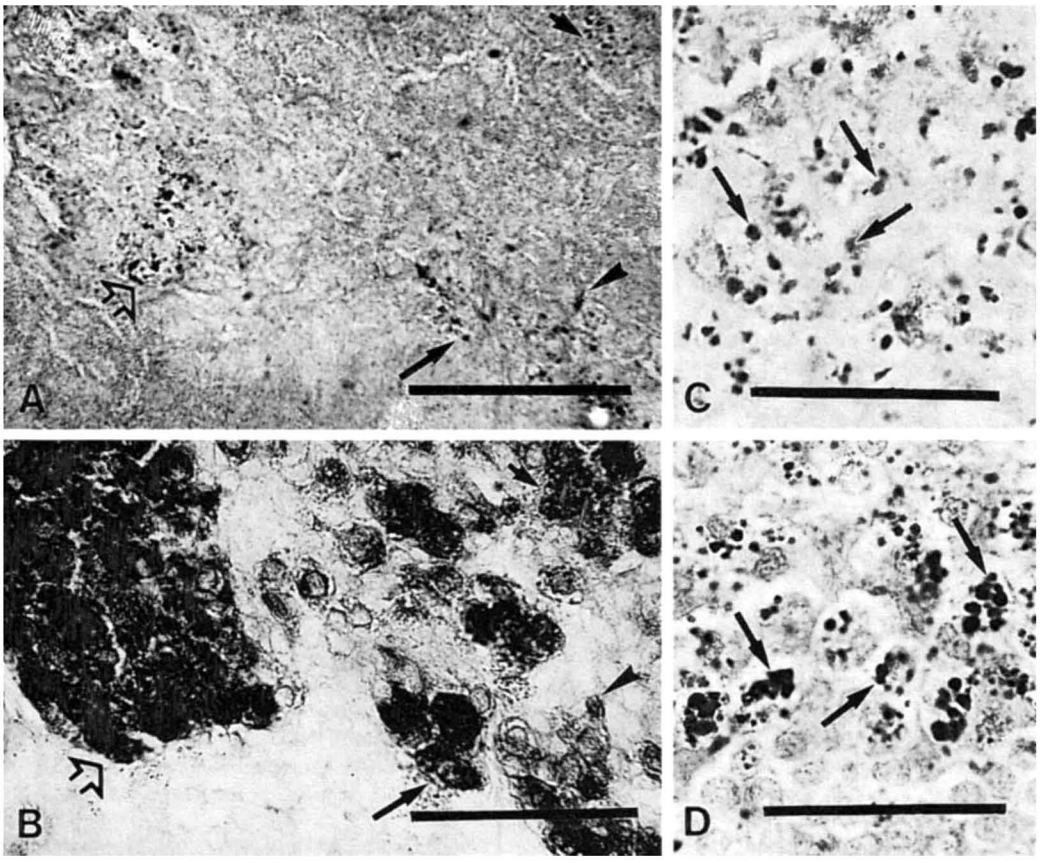

Fig. 3. Sagittal sections of the pituitary gland counterstained with hematoxylin - eosin. A. Proximal pars distalis of a female fish showing carassin-ir granules in some cells (arrow, open arrow, short arrow and arrowhead). B. Corresponding area of the section adjacent to the section shown in A, reacted for GtH. Corresponding arrow, open arrow, short arrow and arrowhead in A and B show that carassin-ir granules are colocalized in a small percentage of GtH cells. C. Male pituitary gland showing cells of the proximal pars distalis (PPD) having a few carassin-ir granules (arrows) in each, as a result of which the granules appear to be sparsely distributed. D. Female pituitary gland showing clusters of granules in some cells of the PPD. Scale bars = 40 μm.

may in turn influence pituitary function. Finally, the demonstration of carassin-ir in GtH cells suggests that it may have an auto- and/or paracrine regulatory function in the pituitary gland. The specific nature of the actions of carassin remain to be investigated.

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